50

IN VITRO KNOCKDOWN OF SOX9 AFFECTS CELL SURVIVAL VIA P21 AND CYCLIN D1 AND FAVORS OSTEOGENIC DIFFERENTIATION OF MSC

S. Stoeckl[1,2], A. Bosserhoff[3], C. Göttl[1,2], J. Grifka[2], S. Grässel[1,2]. [1]*Ctr. for Med. Biotechnology, Regensburg, Germany;* [2]*Orthopaedic Surgery, Experimental Orthopaedics, Regensburg, Germany;* [3]*Dept. of Pathology, Regensburg, Germany*

Purpose: The transcription factor Sox9 regulates skeletal growth and cartilage formation in mesenchymal stem cells and osteochondro-progenitor cells which can differentiate towards chondrocytes or osteoblasts. Sox9 is absent in hypertrophic chondrocytes where a misexpression in vivo causes lack of bone marrow, inhibition of cartilage resorption and failure of vascular invasion in mice. Moreover, there is evidence that Sox9 is related to more biological processes than only to osteo-chondrogenesis, for example cell cycle regulation or tumourigenic development. The aim of our study is to determine the role of Sox9 in undifferentiated rat mesenchymal stem cells (rMSC) in cell survival and its impact on osteogenic differentiation capacity.
Methods: Mesenchymal stem cells (rMSC), isolated from bone-marrow and adipose tissue of 6 week old rats, were transduced with a retroviral vector containing a Sox9-specific shRNA or the Sox9 cDNA sequence for either generating an in vitro gene knockdown or an overexpression of Sox9. The Sox9 level was determined with quantitative real-time PCR (qPCR) on mRNA level and with western blotting and luciferase-reporter assays on protein level. The effects of the altered Sox9 dose on selected marker genes were investigated by qPCR, ELISA and western blotting. Proliferation was quantified with a BrdU – ELISA, cell cycle distribution was analysed with FACS and apoptotic activity with a caspase 3/7 assay. For transduced cells, subjected to osteogenic differentiation for up to 21 days, osteogenic marker gene expression was analyzed via qRT-PCR and osteogenic differentiation status was investigated with alizarin red staining.
Results: A reproducible Sox9 knockdown between 50% and 90% and a 10 to 100 fold overexpression of Sox9 was generated in both, bone marrow (BM) and adipose tissue derived (AD) MSC. Downstream effects of Sox9 silencing are a significant down regulation of Integrin alpha 11, Mmp13 and Bcl-2 gene expression in BM-MSC, whereas Osteocalcin is up regulated in BM-MSC and in AD-MSC. Overexpressing Sox9 results in opposite regulation of gene expression in rBM-MSC. The BrdU assay, measurement of cell doubling time and cell cycle analysis via FACS displays a reduction of proliferation after Sox9 knockdown and a reduced G1 to S-phase transition rate. A caspase 3/7 assay indicates an increase in apoptotic activity. The protein amount of p21 and Cyclin D1, analyzed via western blotting, was clearly increased after Sox9 inhibition. Furthermore, osteogenic differentiation studies demonstrate an upregulation of Runx2, Mmp13, Vegfa and Osteocalcin in Sox9 knockdown cells after 14 days of osteogenesis, whereas Msx2 and Dlx5 expression is diminished.
Conclusions: Based on these studies, we conclude a negative effect of Sox9 on apoptosis, and a positive effect on cell proliferation and cell viability. Alterations of Bcl2, p21 and Cyclin D1 protein expression after Sox9 reduction point to an important role of Sox9 in cell survival and cell cycle control in MSC. Possibly, Sox9 modulates transcriptional activation of inhibitors of p21 and Cyclin D1 or induce phosphorylation through specific kinases and consequently their degradation. Our findings furthermore indicate an acceleration of osteogenic differentiation due to inhibition of Sox9, mainly during early osteogenesis of MSC. A possible mechanism for that effect is a direct or indirect interaction of Sox9 with one of the major transcription (co) factors in early osteogenesis like Runx2, Msx2 or Dlx3, three key players of Osteocalcin expression, which all contain at least one potential Sox9 binding site within their promoter region.

51

FIBROBLAST GROWTH FACTORS 18 AND 9 REGULATE CHONDROGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

D. Correa[1], E. Rom[2], J.F. Welter[1], L. Duesler[1], A. Yayon[2], A.I. Caplan[1]. [1]*Case Western Reserve Univ., Cleveland, OH, USA;* [2]*ProCore Ltd., Ness Ziona, Israel*

Purpose: FGF18 is currently in clinical trials for repair of acute cartilage injury of the knee and in patients with osteoarthritis. Little is known about the role of FGF9 in cartilage biology. This work, therefore, aims to elucidate the effect of these two FGF family members at different stages of the in vitro chondrogenic differentiation program of hMSCs, and the relationship between them and the known mitogenic/pro-chondrogenic effects of FGF2.
Methods: Cell proliferation: coverslip-cultured hMSCs were stimulated for 72 h with 10 ng/ml rhFGF2, rhFGF18 or rhFGF9, followed by BrdU immunostaining. Chondrogenic induction: control and FGF2-expanded cells were cultured in aggregates with (complete chondrogenic medium) and without (defined medium) TGF-β in the presence of 10 ng/ml rhFGF18 or rhFGF9 and mutant ligands that exclusively signal through FGFR3 (FGF18v3 and FGF9v1), starting at different times of the chondroinductive program. qRT-PCR and immunocytochemistry (ICC) was performed at days 0 (right after expansion) and 3, followed by histological/biochemical analysis after 3 weeks. Hypertrophy delay: the aggregate cultures were switched after 2 weeks to a hypertrophy-inducing medium (TGF-β withdrawal, low Dexamethasone, 1 ng/ml Triiodothyronine) for 2 additional weeks, along with rhFGF18, rhFGF9, FGF18v3, FGF9v1, and neutralizing antibodies against FGFR1 and FGFR3. Histological/biochemical analysis and Alkaline Phosphatase (ALP) activity assessment as a marker of hypertrophy-induced changes were then performed.
Results: Cell proliferation: compared with control (24% BrdU-positive cells), the mitogenic effect of FGF2 was confirmed (46%), while FGF18 had no effect (29%) and FGF9 a negative one (15%). Chondrogenic induction: in FGF2-expanded cells Sox9 was upregulated on day 0, suggesting that the chondrogenic regulatory machinery is activated earlier by FGF2. By ICC, Sox9 and BrdU expression did not coincide, suggesting that there are at least two subpopulations which respond differently to FGF2. FGF2 expansion upregulated FGFR2 and FGFR3, while it downregulated FGFR1, evident after three days in aggregate culture with complete chondrogenic medium. FGF18, FGF9 and the mutant ligands (in defined medium) partially replaced TGF-β chondrogenic induction in control-expanded cells, while they inhibited differentiation of FGF2-expanded cells. This suggests that FGFR1 (FGF2) and FGFR2/3 (FGF18/9) signaling antagonize each other, or that these ligands were at "toxic" concentrations, given the upregulated receptor expression generated by FGF2 expansion. FGF18 had an anabolic effect on top of TGF-β when added early (d0). This effect was dissipated when it was administered later (d7 and d14). The effect is mediated by FGFR3, as FGF18v3 recapitulated the same effect. These ligands also had a negative effect on chondrogenesis of FGF2-expanded cells, when administered along with TGF-β, regardless of the time of administration. Hypertrophy delay: FGF18 and FGF9 delay, mainly through FGFR3 signaling, the appearance of the induced hypertrophy-related changes in cell size and ALP activity. Additionally, they still exert a significant anabolic effect (FGF9) even under these special pro-terminally differentiating conditions.
Conclusions: The 3 factors exhibit important effects at all stages of hMSC chondrogenesis. FGF2 expands them, and shifts the chondrogenic program earlier, by upregulating Sox9 expression. FGF18/9, signaling primarily through FGFR3, partially replaces TGF-β, have anabolic properties, and delay the appearance of changes related to hypertrophic differentiation. However, they have a negative impact on FGF2-expanded cells, which requires further research in order to be able to synergize their individual positive effects. Collectively, these factors could be used to optimize the pre-implantation culture conditions of hMSCs when used as part of engineered cartilage grafts.

52

GSK-3ALPHA AND GSK-3BETA MODULATE EARLY STAGES OF CHONDROCYTE DIFFERENTIATION THROUGH PHOSPHORYLATION OF RELA/NF-KAPPAB P65

S. Itoh, T. Saito, M. Hirata, M. Ushita, T. Ikeda, K. Nakamura, U-i. Chung, H. Kawaguchi. *Sensory & Motor System Med., Univ. of Tokyo, Tokyo, Japan*

Purpose: Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase that phosphorylates various substrates to mediate principal signals in many cells. Here we have examined individual roles of the two mammalian isoforms, GSK-3α and GSK-3β, in chondrocytes.
Methods: Chondrocyte differentiation was determined by the mRNA and protein levels of early differentiation markers (Sox9, Col2a1, and aggrecan) and late differentiation markers (Col10a1, Indian hedgehog, and Pth1r). In vitro expression patterns were examined in cultures of mouse chondrogenic ATDC5 cells with the differentiation medium (insulin, transferrin, and sodium selenite) and primary chondrocytes isolated from the ribs of mouse embryos. In vivo expression patterns